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D¹ shock proteins. Upon ligand binding, the receptors dissociate from the inactivating complex and dimerize, which renders them able to bind to DNA and modulate transcription.

Please replace the paragraph on page 2, lines 8-20, with the following:

D² Modified steroid hormone receptors have been developed for use for regulated expression of transgenes (see, *e.g.*, U.S. Patent No. 5,874,534 and published International PCT application No. WO 98/18925, which is based on U.S. provisional application Serial No. 60/029,964) by modifying the ligand specificity of the LBD. In addition, the DNA binding domain of the receptor has been replaced with a non-mammalian DNA binding domain selected from yeast GAL4 DBD, a viral DBD and an insect DBD to provide for regulated expression of a co-administered gene containing a region recognized by the non-mammalian DBD. These constructs, however, have several drawbacks. The non-mammalian DBD is potentially immunogenic and the array of sequences recognized by these DBD is limited, thereby severely restricting gene targets.

Please replace the paragraph on page 2, lines 25-31, with the following:

D³ Polypeptides that function as ligand activated transcriptional regulators and nucleic acid molecules encoding such polypeptides are provided. The polypeptides are fusion proteins that are ligand activated transcriptional regulators that can be targeted to any desired endogenous or exogenous gene. Variants of the fusion protein can be designed to have different selectivity and sensitivity for endogenous and exogenous ligands.

Please replace the paragraph beginning on page 4, line 23, through page 5, line 4, with the following:

Ligand Binding Domain (LBD)

D⁴ The LBD is derived from an intracellular receptor, particularly a steroid hormone receptor. The receptors from which the LBD is derived include, but is not limited to, glucocorticoid receptors, mineralocorticoid receptors, thyroid hormone receptors, retinoic acid receptors, retinoid X receptors, Vitamin D receptors, COUP-TF receptors, ecdysone receptors, Nurr-1 receptors, orphan

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D4
receptors and variants thereof. Receptors of these types include, but are not limited to, estrogen receptors, progesterone receptors, glucocorticoid- α receptors, glucocorticoid- β receptors, androgen receptors and thyroid hormone receptors. LBDs preferably are modified to alter ligand specificity so that they preferentially bind to an exogenous ligand, such as a drug, compared to an endogenous ligand.

Please replace the paragraph on page 5, lines 10-20, with the following:

DS
The LBD is preferably modified so that it does not bind to the endogenous ligand for the receptor from which the LBD is derived, but to a selected ligand to permit fine tuned regulation of targeted genes. Hence, in certain embodiments, the ligand-binding domain has been modified to change its ligand selectivity compared to its selectivity in the native receptor. Preferably the modified ligand-binding domain is not substantially activated by endogenous ligands. Any method for altering ligand specificity, including systematic sequence alteration and testing for specificity, and selection protocols (see, *e.g.*, U.S. Patent No. 5,874,534 and Wang *et al.* (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:8180-8184) can be used.

Please replace the paragraph on page 6, lines 13-19 with the following:

DF
Cys₂His₂ (C2H2) type zinc finger proteins are exemplary of the zinc fingers that can replace the naturally occurring DNA binding domain in an intracellular receptor, such as the C4-C4 type domain in a steroid receptor, to form a functional ligand-responsive transcription factor fusion protein. By virtue of the zinc finger, the resulting fusion protein exhibits altered DNA binding specificity compared to the unmodified intracellular receptor.

Please replace the paragraph on page 6, lines 24-30, with the following:

D2
In preferred embodiments the zinc-finger portion of the fusion protein binds to a nucleotide sequence of the formula (GNN)_n, where G is guanidine, N is any nucleotide and n is an integer from 1 to 6, and typically n is 3 to 6. Preferably, the zinc-finger modular unit is derived from C2H2 zinc-finger peptide.

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D7 More preferably, the zinc-finger peptide is a C2H2 zinc-finger peptide and has at least 90% sequence identity to a human zinc-finger peptide.

Please replace the paragraph on page 7, lines 9-19, with the following:

D8 The transcription regulating domain can be any such domain known to regulate or prepared to regulate eukaryotic transcription. Such TRDs are known, and include, but are not limited to, VP16, VP64, TA2, STAT-6, p65, and derivatives, multimers and combinations thereof that exhibit transcriptional regulation properties. The transcription regulating domain can be derived from an intracellular receptor, such as a nuclear hormone receptor transcription activation (or repression) domain, and is preferably a steroid hormone receptor transcription activation domain or variant thereof that exhibits transcriptional regulation properties. Transcription domains include, but are not limited to, TAF-1, TAF-2, TAU-1, TAU-2, and variants thereof.

Please replace the paragraphs on page 8, lines 1-25, with the following:

Nucleic acid constructs

Also provided are nucleic acid molecules that encode the resulting fusion proteins. The nucleic acids can be included in vectors, suitable for expression of the proteins and/or vectors suitable for gene therapy. Cells containing the vectors are also provided. Typically the cell is a eukaryotic cell. In other embodiments, the cell is a prokaryotic cell.

D9 Also provided are expression cassettes that contain a gene of interest, particularly a gene encoding a therapeutic product, such as an angiogenesis inhibitor, operatively linked to a transcriptional regulatory region or response element, including sequences of nucleic acids to which a fusion protein provided herein binds and controls transcription, particularly upon binding of a ligand to the LBD of the fusion polypeptide. Such expression cassettes can be included in a vector for gene therapy, and are intended for administration with, before or after, administration of the fusion protein or nucleic acid encoding the fusion protein. Genes of interest for exogenous delivery typically encode therapeutic proteins, such as growth factors, growth factor inhibitors or antagonists, tumor

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necrosis factor (TNF) inhibitors, anti-tumor agents, angiogenesis agents, anti-angiogenesis agents, clotting factors, apoptotic and other suicide genes.

Compositions, combinations and kits

D⁹ Also provided are compositions that contain the fusion proteins or the vectors that encode the fusion proteins. Combinations of the fusion proteins or nucleic acids encoding the proteins and nucleic acid encoding a targeted gene with regulatory regions selected for activation by the fusion protein are also provided.

Please replace the paragraph beginning on page 9, line 26, through page 10, line 9, with the following:

Methods

D¹⁰ Methods for regulating expression of endogenous and exogenous genes are provided. The methods are practiced by administering to a cell a composition that contains an effective amount or concentration of the fusion protein or of nucleic acid molecule, such as a vector that encodes the fusion protein. The nucleic acid binding domain (DBD) of the fusion protein is selected to bind to a targeted nucleic acid sequence in the genome of the cell or in an exogenously administered nucleic acid molecule, and the transcription regulating domain (TRD) is selected to regulate transcription from a selected promoter, which typically is operatively linked to the targeted nucleic acid binding domain. The exogenously administered nucleic acid molecule comprises an expression cassette encoding a gene of interest and operatively linked to a regulatory region that contains elements, such as a promoter and response elements.

Please replace the paragraph on page 10, lines 16-21, with the following:

D¹¹ At the same time or at a later time, a composition comprising a ligand that binds to the ligand binding domain of the fusion protein is also administered. The ligand can be administered in the same composition as the fusion protein (or encoding nucleic acid molecule) or in a separate composition.

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D¹¹ The ligand and fusion protein may be administered sequentially, simultaneously or intermittently.

Please replace the paragraphs on page 11, lines 1-29, with the following:

In other embodiments, the methods for regulating gene expression in a cell are effected by administering to the cell a composition containing an effective amount of the nucleic acid molecule that encodes the ligand activated transcriptional regulatory fusion protein, a regulatable expression cassette containing a gene operatively linked to at least one response element for the gene recognized by the nucleotide binding domain of the polypeptide encoded by the polynucleotide, and a pharmaceutically acceptable excipient; and administering to the cell a ligand that binds to the ligand binding domain of the encoded polypeptide, where the nucleotide binding domain of the encoded polypeptide binds to the response element and activates or represses transcription of the gene.

D¹² Methods for treating a cellular proliferative disorder by the *ex vivo* introduction of a recombinant expression vector encoding the fusion protein are provided. Cellular proliferative disorders include disorders associated with transcription of a gene at reduced or increased levels.

Administration of the composition(s) can be effected *in vitro*, *in vivo* or *ex vivo*. One such method includes the removal of a tissue sample from a subject with a disorder, such as a cell proliferative disorder, isolating hematopoietic or other cells from the tissue sample, and contacting isolated cells with the fusion protein or a nucleic acid molecule encoding the fusion protein, and, optionally, a target specific gene. Optionally, the cells can be treated with a growth factor, such as interleukin-2 for example, to stimulate cell growth, before reintroducing the cells into the subject. When reintroduced, the cells specifically target the cell population from which they were originally isolated. In this way, the trans-repressing activity of the zinc finger-nucleotide binding polypeptide may be used to inhibit or suppress undesirable cell proliferation in a subject. Preferably, the subject is a human.

Please replace the paragraph on page 15, lines 3-16, with the following:

D¹³
As used herein, the ligand binding domain (LBD) of the fusion proteins provided herein refers to the portion of the fusion protein responsible for binding to a selected ligand. The LBD optionally and preferably includes dimerization and inactivation functions. The LBDs in the proteins herein are derived from the 300 amino acid carboxyl-terminal half of intracellular receptors, particularly those that are members of the steroid hormone nuclear receptor superfamily. It is the portion of the receptor protein with which a ligand interacts thereby inducing a cascade of events leading to the specific association of an activated receptor with regulatory elements of target genes. In these receptors the LBD includes the hormone binding function, the inactivation function, such as through interactions with heat shock proteins (hsp), and dimerization function. The LBDs used herein include such LBDs and modified derivatives thereof, particularly forms with altered ligand specificity.

Please replace the paragraphs beginning on page 15, line 24, through page 16, line 17, with the following:

D¹⁴
As used herein, the DNA binding domain (DBD), or alternatively the nucleic acid (or nucleotide) binding domain, refers to the portion of the fusion polypeptide provided herein that provides specific nucleic acid binding capability. The use of the abbreviation DBD is not meant to limit it to DNA binding domains, but is also intended to include polypeptides that bind to RNA. The nucleic acid binding domain functions to target the protein to specific genes by virtue of the specificity of the interaction of the TRD region for nucleotide sequences operatively linked to the transcriptional apparatus of a gene. The DBD targets the fusion protein to the selected targeted gene or genes, which gene(s) may be endogenous or exogenously added.

As used herein, operatively linked means that elements of the fusion polypeptide, for example, are linked such that each perform or function as intended. For example, the repressor is attached to the binding domain in such a manner that, when bound to a target nucleotide via that binding domain, the

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D¹⁴
repressor acts to inhibit or prevent transcription. Linkage between and among elements may be direct or indirect, such as via a linker. The elements are not necessarily adjacent. Hence a repressor domain of a TRD can be linked to a DNA binding domain using any linking procedure well known in the art. It may be necessary to include a linker moiety between the two domains. Such a linker moiety is typically a short sequence of amino acid residues that provides spacing between the domains. So long as the linker does not interfere with any of the functions of the binding or repressor domains, any sequence can be used.

Please replace the paragraphs beginning on page 17, line 12, through page 18, line 8, with the following:

D¹⁵
In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. co., p.224).

As used herein, a delivery plasmid is a plasmid vector that carries or delivers nucleic acids encoding a therapeutic gene or gene that encodes a therapeutic product or a precursor thereof or a regulatory gene or other factor that results in a therapeutic effect when delivered *in vivo* in or into a cell line, such as, but not limited to a packaging cell line, to propagate therapeutic viral vectors.

As used herein, "recombinant expression vector" or "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of heterologous DNA, such as nucleic acid encoding the fusion proteins herein or expression cassettes provided herein. Such expression vectors contain a promoter sequence for efficient transcription of the inserted nucleic acid in a cell. The expression vector typically contains an

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origin of replication, a promoter, as well as specific genes that permit phenotypic selection of transformed cells.

D¹⁵
As used herein, a DNA or nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a sequence encoding a therapeutic polypeptide. By the term "substantially homologous" is meant having at least 80%, preferably at least 90%, most preferably at least 95% homology therewith or a less percentage of homology or identity and conserved biological activity or function.

Please replace the paragraphs on page 19, lines 1-30, with the following:

Whether any two nucleic acid molecules have nucleotide sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:2444. Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine identity.

D¹⁶
In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques (see, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo *et al.* (1988) *SIAM J Applied Math* 48:1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Hugu Computers,

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Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo *et al.* (1988) *SIAM J Applied Math* 48:1073. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al.*, *Nucleic Acids Research* 12(II):387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F., *et al.*, *J Molec Biol* 215:403 (1990)).

Please replace the paragraphs beginning on page 20, line 28, through page 22, line 2, with the following:

D¹⁷
As used herein, genetic therapy involves the transfer of heterologous DNA to the certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The DNA is introduced into the selected target cells in a manner such that the heterologous DNA is expressed and a therapeutic product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product, or it may encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy may also be used to deliver nucleic acid encoding a gene product that replaces a defective gene or supplements a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid may encode a therapeutic compound, such as a growth factor inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous DNA encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof. Genetic therapy may also involve delivery of an inhibitor or repressor or other modulator of gene expression.

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D¹⁷
As used herein, heterologous DNA is DNA that encodes RNA and proteins that are not normally produced *in vivo* by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

Please replace the paragraph on page 23, lines 1-16, with the following:

D¹⁸
As used herein, isolated with reference to a nucleic acid molecule or polypeptide or other biomolecule means that the nucleic acid or polypeptide has separated from the genetic environment from which the polypeptide or nucleic acid were obtained. It may also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of a compound can be substantially purified by the one-step method described in Smith *et al.* (1988) *Gene* 67:31-40. The terms isolated and purified are sometimes used interchangeably.

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Please replace the paragraph beginning on page 23, line 24, through page 24, line 2, with the following:

D¹⁹
Isolated or purified as it refers to preparations made from biological cells or hosts means any cell extract containing the indicated DNA or protein including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the DNA or protein of interest can be present at various degrees of purity in these preparations. The procedures may include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

Please replace the paragraph on page 28, lines 14-23, with the following:

D²⁰
As used herein with regard to nucleic acid molecules, including DNA fragments, the phrase "operatively linked" means the sequences or segments have been covalently joined, preferably by conventional phosphodiester bonds, into one strand of DNA, whether in single or double stranded form such that operatively linked portions function as intended. The choice of vector to which a transcription unit or a cassette provided herein is operatively linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules.

Please replace the paragraph on page 29, lines 26-30, with the following:

D²¹
As used herein, ligand refers to any compound that interacts with the ligand binding domain of a receptor and modulates its activity; ligands typically activate receptors. Ligand can also include compounds that activate the receptor without binding. A natural ligand is a compound that normally interacts with the receptor.

Please replace the paragraph on page 30, lines 4-11, with the following:

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D²² As used herein, non-natural ligands or non-native ligands refer to compounds that are normally not found in mammals, such as humans, that bind to or interact with the ligand binding domain of a receptor. Hence, the term "non-native ligands" refers to those ligands that are not naturally found in the specific organism (man or animal) in which gene therapy is contemplated. For example, certain insect hormones such as ecdysone are not found in humans. As such ecdysone is non-native hormone to an animal, such as a human.

Please replace the paragraph on page 31, lines 1-4, with the following:

D²³ As used herein, administration of a therapeutic composition can be effected by any means, and includes, but is not limited to, subcutaneous, intravenous, intramuscular, intrasternal, infusion techniques, intraperitoneal administration and parenteral administration.

Please replace the paragraphs beginning on page 31, line 22, through page 32, line 11, with the following:

D²⁴ The fusion protein also includes a LBD that is derived from an intracellular receptor, preferably a hormone receptor, more preferably a steroid receptor. The LBD can be modified to have altered ligand specificity so that endogenous or natural ligands do not interact with it, but non-natural ligands do. The fusion protein also can include a transcription regulating domain (TRD) that regulates transcription of the targeted gene(s). In some embodiments, the TRD can repress transcription of an endogenous gene; in others it can activate expression of an endogenous or exogenous gene.

Hence the fusion protein is made by operably linking a LBD domain from an intracellular receptor to one or more zinc finger domains, selected to bind to a targeted gene. A transcription regulating domain can also be operably linked. This is accomplished by any method known to those of skill in the art. Generally the fusion protein is produced by expressing nucleic acid encoding the fusion protein.

1. Ligand Binding Domain (LBD)

D²⁴
The ligand binding domain is derived from an intracellular receptor, and is preferably derived from a nuclear hormone receptor. The LBD of an intracellular receptor includes the approximately 300 amino acids from the carboxy terminus, which can be used with or without modification.

Please replace the paragraphs beginning on page 32, line 28, through page 33, line 25, with the following:

D²⁵
The LBD can be modified by deletion of from about 1 up to about 150, typically 120, amino acids on the carboxyl terminal end of the receptor from which the LBD derives. Systematic deletion of amino acids and subsequent testing of the ligand specificity and of the resulting LBD can be used to empirically identify mutations that lead to modified LBDs that have desired properties, such as preferential interaction with non-natural ligands. Exemplary mutations are described in the Examples herein, and also are known to those of skill in the art (see, *e.g.*, U.S. Patent No. 5,874,534; U.S. Patent No. 5,935,934; U.S. Patent No. 5,364,791; and International PCT application No. 98/18925, which is based on U.S. provisional application Serial No. 60/029,964; International PCT application No. 96/40911, which is based on U.S. application Serial No. 08/479,9131) and references cited therein. Hence a LBD or modified form thereof prepared by known methods is obtained and operably linked to a DBD; a TRD is also linked as needed.

2. Nucleic Acid Binding Domain (DBD)

Zinc fingers are modular nucleic acid binding peptides. The zinc fingers, or modules thereof, or variants thereof can be used to construct fusion proteins that specifically interact with targeted sequences. Zinc fingers are ubiquitous proteins, and many are well-characterized. For example, methods and rules for preparation and selection of zinc fingers based upon the C2H2 class of zinc fingers with unique specificity are known (see, *e.g.*, International PCT application No. WO 98/54311 and International PCT application No. 95/19431; see, also U.S. Patent No. 5,789,538; Beerli *et al.* (1999) *Proc. Natl. Acad. Sci.*

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U.S.A. 96:2758-2763; Beerli *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633; see, also U.S. application Serial No. 09/173,941, filed 16 October, 1998, published as International PCT application No. WO 00/23464). Exemplary targeting sequences are provided herein.

Please replace the paragraph beginning on page 34, line 16, through page 35, line 2, with the following:

D²⁶
For example, zinc finger variants can be prepared by identifying a zinc finger or modular unit thereof, creating an expression library, such as a phage display library (see, *e.g.*, International PCT application No. WO 98/54311, Barbas *et al.* (1991) *Methods* 2:119; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:4457), encoding polypeptide variants of the zinc finger or modular units thereof, expressing the library in a host and screening for variant peptides having a desired specificity. Zinc fingers may also be constructed by combining amino acids (or encoding nucleic acids) according to the known rules of binding specificity and, if necessary, testing or screening the resulting peptides to ensure the peptide has a desired specificity. Because of the modular nature of zinc fingers, where each module can be prepared to bind to a three nucleotide sequence, peptides of any specificity can be prepared from the modules. The number of modules used depends upon the specificity of gene targeting desired. Modular units are combined; spacers (*i.e.* TGEKP, TGQKP) required to maintain spacing and conformational features of the modular domains are included in the peptide (see, *e.g.*, WO 98/54311).

Please replace the paragraph on page 36, lines 5-13, with the following:

D²⁷
A zinc finger-nucleotide binding peptide domain contains a unique heptamer (contiguous sequence of 7 amino acid residues) within the α -helical domain of the polypeptide, which heptameric sequence determines binding specificity to a target nucleotide. The heptameric sequence can be located anywhere within the α -helical domain but it is preferred that the heptamer extend from position -1 to position 6 as the residues are conventionally numbered in the art. A peptide nucleotide-binding domain can include any β -

D²⁷ sheet and framework sequences known in the art to function as part of a zinc finger protein.

Please replace the paragraph on page 40, lines 15-21, with the following:

D²⁸ More often, however, two or three amino acids are selected for nucleotide recognition. His3 or Lys3 (and to a lesser extent, Gly3) are selected for the recognition of a middle guanine. Ser3 and Ala3 are selected to recognize a middle thymine. Thr3, Asp3, and Glu3 are selected to recognize a middle cytosine. Asp and Glu are selected in position -1 to recognize a 3' cytosine, while Thr-1 and Ser-1 are selected to recognize a 3' thymine.

Please replace the paragraph on page 41, lines 13-23, with the following:

D²⁹ Further the data demonstrate that sequence motifs at positions -1, 1, and 2 rather than the simple identity of the position 1 residue are required for highly specific recognition of the 3' base. These residues likely provide the proper stereo-chemical context for interactions of the helix in terms of recognition of specific bases and in the exclusion of other bases, the net result being highly specific interactions. Ready recombination of the disclosed domains then allows for the creation of proteins, typically polydactyl proteins, of defined specificity precluding the need to develop phage display libraries in their generation. Such family of zinc finger domains is sufficient for the construction of 16 or 17 million proteins that bind to the 5'-(GNN)₆-3' family of DNA sequences.

Please replace the paragraphs beginning on page 45, line 16, through page 46, line 19, with the following:

e. Screening of variant zinc finger and other DBD peptides

D³⁰ Any method known to those of skill in the art for identification of functional modular domains derived from zinc fingers and combinations thereof can be employed. An exemplary method for identifying variants of zinc fingers or other polypeptides that bind to zinc finger binding motifs is provided. Components used in the method include a nucleic acid molecule encoding a putative or modified zinc finger peptide operably linked to a first inducible promoter and a reporter gene operably linked to a second inducible promoter

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and a zinc finger-nucleotide binding motif, wherein the incubating is carried out under conditions sufficient to allow the components to interact, and measuring the affect of the putative DBD peptide on the expression of the reporter gene is provided.

D³⁰ For example, a first inducible promoter, such as the arabinose promoter, is operably linked to the nucleotide sequence encoding the putative DBD polypeptide. A second inducible promoter, such as the lactose promoter, is operably linked to a zinc finger derived-DNA binding motif followed by a reporter gene, such as β -galactosidase. Incubation of the components may be *in vitro* or *in vivo*. *In vivo* incubation may include prokaryotic or eukaryotic systems, such as *E.coli* or COS cells, respectively. Conditions that allow the assay to proceed include incubation in the presence of a substance, such as arabinose and lactose, which activate the first and second inducible promoters, respectively, thereby allowing expression of the nucleotide sequence encoding the putative trans-modulating protein nucleotide sequence. Determination of whether the putative modulating protein binds to the zinc finger-nucleotide binding motif, which is operably linked to the second inducible promoter, and affects its activity is measured by the expression of the reporter gene. For example, if the reporter gene is β -galactosidase, the presence of blue or white plaques indicates whether the putative modulating protein enhances or inhibits, respectively, gene expression from the promoter. Other commonly used assays to assess the function from a promoter, including chloramphenicol acetyl transferase (CAT) assay, are known to those of skill in the art. Prokaryote and eukaryote systems can be used.

Please replace the paragraph beginning on page 50, line 20, through page 51, line 2, with the following:

D³¹ Transcriptional repressors are well known in the art, and any such repressor can be used herein. The repressor is a polypeptide that is operatively linked to the nucleic acid binding domain as set forth above. The repressor is operatively linked to the binding domain in that it is attached to the binding

D 31
domain in such a manner that, when bound to a target nucleotide via that binding domain, the repressor acts to inhibit or prevent transcription. The repressor domain can be linked to the binding domain using any linking procedure well known in the art. It may be necessary to include a linker moiety between the two domains. Such a linker moiety is typically a short sequence of amino acid residues that provides spacing between the domains. So long as the linker does not interfere with any of the functions of the binding or repressor domains, any sequence can be used.

Please replace the paragraphs on page 52, lines 1-23, with the following:

c. **Activators**

D 32
Exemplary and preferred transcription activation domains include any protein or factor that regulates transcription. Exemplary transcriptional regulation domains include, but are not limited to, VP16, TA2, VP64, STAT6 and relA.

4. **Exemplary construct based on human integrin $\beta 3$ and erbB-2 target sequences**

To exemplify the generation of zinc finger modular domains and peptides containing one or more of such domains to produce peptides with DNA binding specificity and therapeutic potential, target sequences have been identified based on human integrin $\beta 3$ and *erbB-2* (Ishii *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:4374-4378) genomic sequences.

Integrin $\beta 3$ as a target for cancer gene therapy

Integrin $\alpha_v\beta_3$ is the most promiscuous member of the integrin family and has been identified as a marker of angiogenic vascular tissue. For instance, integrin $\alpha_v\beta_3$ shows enhanced expression on blood vessels in human wound granulation tissue but not in normal skin. Following the induction of angiogenesis, blood vessels show a four-fold increase in $\alpha_v\beta_3$ expression compared to blood vessels not undergoing this process. It has been reported that a cyclic peptide or monoclonal antibody antagonist of integrin $\alpha_v\beta_3$ blocks cytokine- or tumor-induced angiogenesis on the chick chorioallantoic membrane.

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D³² Therefore, inhibition of integrin $\alpha_v\beta_3$ expression provides an approach to block tumor-induced angiogenesis.

Please replace the paragraph beginning on page 55, line 22, through page 56, line 4, with the following:

D³³ In another embodiment, gene therapy can be accomplished using a combination of the vectors described above. For example, a retroviral vector can deliver a stably integrated, inducible transgene cassette into a population of cells either *in vitro* (*ex vivo*) or *in vivo*. Subsequently, the integrated transgene can be activated by transducing this same cell population with a second vector, such as an adenovirus vector capable of expressing the fusion protein, followed by the administration of the specific ligand inducing agent. This is particularly useful where "one time" activation of the transgene is desired, for example as a cellular suicide mechanism. An example of this application is the stable integration of an inducible transgene cassette containing the herpes simplex virus thymidine kinase gene (HSV Tk). Subsequent activation of this gene confers sensitivity to ganciclovir and allows ablation of this modified cell.

Please replace the paragraphs beginning on page 57, line 20, through page 58, line 12, with the following:

D³⁴ Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include but are not limited to $\Psi 2$, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced. The vector virions produced by this

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method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

b. Nonviral Delivery systems

³⁴
D "Non-viral" delivery techniques for gene therapy include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA, CaPO₄ precipitation, gene gun techniques, electroporation, liposomes and lipofection. Any of these methods are available to one skilled in the art and would be suitable for use herein. Other suitable methods are available to one skilled in the art, and it is to be understood that the methods herein may be accomplished using any of the available methods of transfection.

Please replace the paragraph on page 60, lines 14-18, with the following:

³⁵
D In general, the compounds bound to the surface of the targeted delivery system are ligands and receptors permitting the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest that interacts with another compound, such as a receptor.

Please replace the paragraph on page 61, lines 15-25, with the following:

³⁶
D Administration of nucleic acid molecules provided herein to a target cell *in vivo* may be accomplished using any of a variety of techniques well known to those skilled in the art. The vectors of the methods herein may be administered orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable non-irritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature and therefore melt in the rectum and release the drug.

Please replace the paragraphs beginning on page 62, line 27, through page 63, line 27, with the following:

3. Ligands

As noted, the ligands may be naturally-occurring ligands, but are preferentially non-natural ligands with which the LBD is modified to specifically interact. Methods for modifying the LBD are known, as are methods for screening for such ligands.

D³⁷
Ligands include, non-natural ligands, hormones, anti-hormones, synthetic hormones, and other such compounds. Examples of non-natural ligands, anti-hormones and non-native ligands include, but are not limited to, the following: 11 β -(4-dimethylaminophenyl)-17 α -hydroxy-17 α -propinyl-4,9-estradiene-3-one (RU38486 or Mifepristone); 11 β -(4-dimethylaminophenyl)-17 α -hydroxy-17 β -(3-hydroxypropyl)-13 α -methyl-4,9-gonadiene-3-one (ZK98299 or Onapristone); 11 β -(4-acetylphenyl)-17 β -hydroxy-17 α -(1-propinyl)-4,9-estradiene-3-one (ZK112993); 11 β -(4-dimethylaminophenyl)-17 β -hydroxy-17 α -(3-hydroxy-1 (Z)-propenyl-estra-4,9-diene-3-one (ZK98734); (7 β 11 β ,17 β)-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ester-4,9-diene-17,2' (3'H)-furan]-3-one (Org31806); (11 β ,14 β ,17 α)-4',5'-dihydro-11-(4-dimethylaminophenyl)-y'spiroestra-4,9-diene-17,2' (3'H)-furan]-3-one (Org31376); 5-alpha-pregnane-3,2-dione. Additional non-natural ligands include, in general, synthetic non-steroidal estrogenic or anti-estrogenic compounds, broadly defined as selective estrogen receptor modulators (SERMS). Exemplary compounds include, but are not limited to, tamoxifen and raloxifen.

4. Pharmaceutical compositions and combinations

Also provided is a pharmaceutical composition containing a therapeutically effective amount of the fusion protein, or a nucleic acid molecule encoding the fusion protein in a pharmaceutically acceptable carrier. Pharmaceutical compositions containing one or more fusion proteins with different zinc finger-nucleotide binding domains are contemplated. Also provided are pharmaceutical compositions containing the expression cassettes,

D 37
and also compositions containing the ligands. Combinations containing a plurality of compositions are also provided.

Please replace the paragraph on page 65, lines 5-15, with the following:

D. Methods of gene regulation

D 38
Methods of regulating expression of endogenous and exogenous genes are provided. In particular, ligand-dependent methods are provided. In practicing the methods, a target nucleic acid molecule containing a sequence that interacts with the nucleic acid binding domain of the fusion protein exposed to an effective amount of the fusion protein in the presence of an effective binding amount of a ligand, which can be added simultaneous with or subsequent to the fusion protein. The nucleic acid binding domain of the fusion protein binds to a portion of the target nucleic acid molecule and the ligand binds to the ligand binding domain of the fusion protein. Exposure can occur *in vitro*, *in situ* or *in vivo*.

Please replace the paragraph beginning on page 66, line 11, through page 67, line 2, with the following:

Treatments

D 39
Methods for gene therapy are provided. The fusion proteins are administered either as a protein or as a nucleic acid encoding the protein and delivered to cells or tissues in a mammal, such as a human. The fusion protein is targeted either to a specific sequence in the genome (an endogenous gene) or to an exogenously added gene, which is administered as part of an expression cassette. Prior to, simultaneous with or subsequent to administration of the fusion protein, a ligand that specifically interacts with the LBD in the fusion protein is administered. In embodiments, in which the targeted gene is exogenous, the expression cassette, which can be present in a vector, is administered, simultaneous with or subsequent to administration of the fusion protein. These methods are intended for treatment of any genetic disease, for treatment of acquired disease and any other conditions. Diseases include, cell proliferative disorders, such as cancer. Such therapy achieves its therapeutic

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D³⁹
effect by introduction of the fusion protein that includes the zinc finger-nucleotide binding polypeptide, either as the fusion protein or encoded by a nucleic acid molecule that is expressed in the cells, into cells of animals having the disorder. Delivery of the fusion protein or nucleic acid molecule can be effected by any method known to those of skill in the art, including methods described herein. For example, it can be effected using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system.

Please replace the paragraph beginning on page 74, line 17, through page 75, line 2, with the following:

Selection strategy for the generation of six-finger proteins with DNA binding specificity

D⁴⁰
Based on the modular nature of zinc finger domains, as well as the fact that each zinc finger recognizes 3 bp of DNA sequence, several strategies can be employed to generate zinc finger proteins, with preferably one to three fingers, with desired DNA binding specificity. For instance, *in vitro* evolution of a six-finger protein binding an 18bp target sequence can follow the strategy outlined in FIGURE 1. The target sequence is divided into six 3bp sub-sites, A-F. In the first step, a Zif268-based zinc finger phage display library in which the central finger 2 is randomized is selected against all 6 subsites in the context of the 2 wild type fingers. After successful generation of all the finger 2 variants required for a given target, cDNAs encoding three-finger proteins recognizing either half-site 1 (ABC) or half-site 2 (DEF) are constructed via PCR overlap extension. Finally, standard cloning procedures are used to construct a gene encoding a six-finger protein recognizing the whole 18bp target site.

Please replace the paragraph on page 84, lines 1-15, with the following:

EXAMPLE 2

Construction Of Fusion Proteins Containing Zinc Finger Domains and Transcriptional Repressors And Activators

D⁴¹
In order to demonstrate use of zinc finger proteins as gene-specific transcriptional regulators, the E2C(Sp1), B3B(Sp1), and B3C2(Sp1) six-finger

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D 41 proteins were fused to a number of effector domains (Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). Transcriptional repressors were generated by attaching either of three human-derived repressor domains to the zinc finger protein. The first repressor protein was prepared using the ERF repressor domain (ERD) (Sgouras *et al.* (1995) *EMBO J.* 14:4781-4793), defined by amino acids 473 to 530 of the *ets2* repressor factor (ERF). This domain mediates the antagonistic effect of ERF on the activity of transcription factors of the *ets* family. A synthetic repressor was constructed by fusion of this domain to the C-terminus of the zinc finger protein.

Please replace the paragraph beginning on page 85, line 7, through page 86, line 6, with the following:

*Specific regulation of *erbB-2* promoter activity*

D 42 Reporter constructs containing fragments of the *erbB-2* promoter coupled to a luciferase reporter gene were generated to test the specific activities of the *erbB-2* specific synthetic transcriptional regulators. The target reporter plasmid contained nucleotides -758 to -1 with respect to the ATG initiation codon, whereas the control reporter plasmid contained nucleotides -1571 to -24, thus lacking all but one nucleotide of the E2C binding site encompassed in positions -24 to -7. Both promoter fragments displayed similar activities when transfected transiently into HeLa cells, in agreement with previous observations. To test the effect of zinc finger-repressor domain fusion constructs on *erbB-2* promoter activity, HeLa cells were transiently co-transfected with each of the zinc finger expression vectors and the luciferase reporter constructs (Beerli *et al.*, (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). Significant repression was observed with each construct. The ERD and SID fusion proteins produced approximately 50% and 80% repression, respectively. The most potent repressor was the KRAB fusion protein. This protein caused complete repression of *erbB-2* promoter activity. The observed residual activity was at the background level of the promoter-less pGL3 reporter. In contrast, none of the proteins caused significant repression of the control *erbB-2* reporter construct

D⁴²
lacking the E2C target site, demonstrating that repression is indeed mediated by specific binding of the E2C(Sp1) protein to its target site. Expression of a zinc finger protein lacking any effector domain resulted in weak repression, approximately 30%, indicating that most of the repression observed with the SID and KRAB constructs is caused by their effector domains, rather than by DNA-binding alone. This observation strongly suggests that the mechanism of repression is active inhibition of transcription initiation rather than of elongation. Once initiation of transcription by RNA polymerase II has occurred, the zinc finger protein appears to be readily displaced from the DNA by the action of the polymerase.

Please replace the paragraph on page 86, lines 17-25, with the following:

D⁴³
Based on the efficient and specific regulation of a reporter construct driven by the *erbB-2* promoter, the effect of transiently transfected zinc finger expression plasmids on activity of the endogenous *erbB-2* promoter was analyzed. As a read-out of *erbB-2* promoter activity, ErbB-2 protein levels were analyzed by Western blotting. Significantly, E2C(Sp1)-VP64 lead to an upregulation of ErbB-2 protein levels, while E2C(Sp1)-SKD lead to its downregulation. This regulation was specific, since no effect was observed on expression of EGFR.

Please replace the paragraph on page 87, lines 5-17, with the following:

Specific regulation of integrin $\beta 3$ promoter activity

D⁴⁴
To test the activity of transcriptional regulators specific for the integrin $\beta 3$ promoter, a reporter plasmid was constructed containing the luciferase open reading frame under control of the integrin $\beta 3$ promoter. When compared to the two *erbB-2* promoter fragments described above, the integrin $\beta 3$ promoter fragment had a very low activity. In fact, in some experiments no activation of luciferase expression over background was detected, preventing an analysis of the effects of the KRAB fusion proteins. However, when the VP64 fusion proteins were tested an efficient activation of the integrin $\beta 3$ promoter was observed. B3B(Sp1)-VP64 and B3C2(Sp1)-VP64 stimulated transcription 12 and

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D44 22-fold, respectively. Activation of transcription was specific, since no effect on the activity of the *erbB-2* promoter was detected.

Please replace the paragraph on page 88, lines 3-9, with the following:

D45 The original PR DNA binding domain can be replaced by engineered zinc finger proteins. For example, the three finger protein Zif268(C7) was fused to the N-terminus of the PR ligand binding domain (PBD) (aa 640 to 914), and the VP16 activation domain to its C-terminus. It was found that this fusion protein was able to regulate an SV40 promoter luciferase construct with ten upstream Zif268(C7) binding sites in an RU486-dependent manner.

Please replace the paragraph beginning on page 89, line 30, through page 90, line 10, with the following:

D46 In order to optimize the ability of the fusion proteins to regulate gene expression, it may be necessary to add additional heterologous transactivating domains to the receptor. To facilitate these studies, fusion proteins were constructed either with the full length LBD extending to estrogen receptor residue 595, or with LBD fragments truncated at amino acid (aa) 554 to remove the F region. The full-length constructs are referred to as long (L), the truncated versions as short (S). All constructs contain a heterologous transactivation domain (TA) comprised of a VP16 minimal domain, unless otherwise noted, fused to the carboxy terminus of the ligand binding domain. VP16 minimal domain trimer has the amino acid residue sequence 3 x (PADALDDFDLDMML) (SEQ ID NO: 47), and is the tetracycline controlled transactivator (tTA) TA2 (Baron *et al.* (1997) *Nucleic Acids Research* 25:2723-2729).

Please replace the paragraph beginning on page 91, line 10, through page 92, line 3, with the following:

D47 The general cloning strategy was as follows. Three fragments (A, B, and C with reference to FIG. 3) of human estrogen receptor ligand binding domain (LBD) with or without the F region were built into the pcDNA3.1 (Invitrogen) vector backbone through a series of PCR amplification and cloning steps. Initially the LBD fragment A without F region (i.e. short form; LBDAS) and with

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D⁴⁷
F region (i.e. long form; LBDAL) were PCR amplified from a plasmid clone of the human wild type estrogen receptor, pHEGO (Tora *et al.* *EMBO J.* 8:1981-1986) with primer pairs NR1/NR2 and NR1/NR3 respectively (Table 1). Convenient restriction sites were incorporated into primers (Table 1) as needed. The PCR amplified LBDAS and LBDAL fragments were first cloned into the *Srf*I site of pCR-ScriptAmpSK(+) vector (Stratagene), resulting in constructs pLBDAS and pLBDAL. The VP16 minimal domain trimer (TA2; Baron *et al.* (1997) *Nucleic Acids Research* 25:2723-2729) was PCR amplified from plasmid pTTA2 (Clontech) with primer pairs NR4 and NR9 and cloned into the *Sp*I and *Not*I site of pLBDAS and pLBDAL to generate pLBDASTA2 and pLBDALTA2. To generate LBD fragment B without the F region (LBDBS) and LBD fragment C without the F region (LBDCS), PCR primers NR7 and NR8, which represent the 5' boundary of the LBD region fragment in chimerics B and C respectively were designed (Table 6, below). These primers were paired with the 3' end primer NR6, which incorporates a unique *B*/pI site in ER. PCR fragments from pHEGO with primer pair NR6/NR7 and PCR fragment with NR6/NR8 were then cloned into the *Spe*I and *B*/pI site of pLBDC7ASTA2 backbone. This resulted in plasmid pLBDBSTA2 and pLBDCSTA2.

Please replace the paragraphs beginning on page 94, line 3, through page 95, line 16, with the following:

D⁴⁸
A DNA oligonucleotide containing two inverted binding sites for the C2H2 domain known as C7, each half site separated by 3 bp, was used for the initial assessment of DNA binding. This palindromic configuration mimics the composition of the native estrogen receptor response element (ERE), except that the natural 6 bp half site of ERE is replaced by the 9 bp half site specified by C7. Binding of the C7-LBD fusion proteins A, B, and C, all in the short form, were tested and compared to the control proteins C7VP16 and 2C7VP16 (see, Liu, *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A* 94:5525-5530, which describes the control proteins). For each protein, binding was tested in the absence or presence of 100 fold excess of unlabeled oligonucleotide (1.75 μ M) as a

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competitor. Competition of the gel shift product by the unlabeled oligonucleotide indicates the band is a specific protein:DNA interaction. The results demonstrated that C7VP16 can bind once or twice to the oligonucleotide, creating two specific gel shift bands. 2C7VP16 binds only once to the oligonucleotide containing two inverted C7 sites. Notably, C7LBDA and C7LBDB bind strongly to yield one major species, which runs higher than any of the control bands. Although true molecular mass cannot be determined from this type of mobility assay, the relative size of the complexes suggest the protein bound for C7LBD is larger than for C7VP or 2C7VP. The size of the band and presence of only one major species indicate that the fusion protein ZFP-LBD is binding to the oligonucleotide as a dimer. No significant gel shift product was detected for C7LBD chimeric C, suggesting that the addition of the additional native zinc finger from the estrogen receptor may have reduced the affinity of the fusion protein for its C2H2-specific DNA binding site. Finally, the reduction of binding for each of the gel shift products by the addition of the unlabeled oligonucleotide indicates that these fusion proteins are binding to DNA in a sequence specific manner.

To further demonstrate that the chimera ZFP-LBD binds to DNA as a dimer, the binding of C7LBD A, B, and C to oligonucleotides containing one or two C7 binding sites was tested. Three fusion proteins (C7LBDAS, C7LBDBS and C7LBDCS) were tested against three different target oligonucleotide sequences, which contained one C7 half site or two C7 half sites either in palindromic or direct repeat orientation.

Oligo 1: gat cca aag tcg cgt ggg cgc agc gcc cac gcg atc aaa ga (SEQ ID NO: 48)

Oligo 2: gat cca aag tcc agg cga gcg cgt ggg cgg cag atc aaa ga (SEQ ID NO: 49)

Oligo 3: gat cca aag tcg cgt ggg cgc agg cgc gag cgt ggg cgg atc aaa ga (SEQ ID NO: 50)

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D⁴⁸
Gel shift assay conditions were the same as the standard protocol described above. The results showed that C7LBDAS and C7LBDBS were able to bind to both oligonucleotides containing two C7 half sites, but not to the oligo containing only one half site. C7LBDCS bound weakly or not at all to all three targets.

Please replace the paragraph on page 98, lines 15-25, with the following:

D⁴⁹
The ability of C7LBD short form chimeric proteins A, B, and C to regulate reporter gene expression in an estrogen-dependent manner was studied in Cos and HeLa cells. The constitutive activators C7VP16 and 2C7VP16 were used as positive controls. The results show that the three ZFP-LBD fusion proteins gave a similar profile in Cos and HeLa cells. All three ZFP-LBD fusion proteins had an estrogen dependent effect on the luciferase reporter gene. The characteristic pattern is that A has greater total activity than B and B has greater total activity than C. Likewise, the basal or ligand-independent effect of these proteins on the reporter gene follows a similar pattern: A > B > C. The estrogen dependent effect on gene expression ranged from two-fold to nine-fold in these experiments.

Please replace the paragraphs beginning on page 99, line 28, through page 100, line 8, with the following:

D⁵⁰
Assays were performed with HeLa cells transfected with 0.5 ug of 6x2C7pGL3Luc reporter and 0.1 μ g regulator, Luc activity was determined as previously described. When the human STAT6 transactivation domain was used to replace the TA2 VP minimal domain trimer, the same low basal activity and 9 fold ligand dependent induction of transgene, two-fold less than with the TA2 domain, was obtained.

The incorporation of NLS upstream of the full length VP16 (FIG. 24, C7ASnlsVP16) greatly increased the folding induction compared to TA2 or VP16 without the NLS, but the total activity was significantly decreased. When the full length VP16 domain was used, it gave about 2 fold higher total activity, but high basal activity resulting in weaker ligand dependent induction (3-fold).

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Please replace the paragraph on page 109, lines 16-24, with the following:

DS1
All four fusion constructs were fully sequenced and tested in a luciferase assay for their ability to regulate the *erbB-2* promoter in a ligand-dependent manner. It was found that the PR 6 Finger heterodimer was inactive; a similar observation was made with an C7-RxR // EcR-VP16 heterodimer. In contrast, the E2C-ER // ER-VP64 heterodimer had some activity, and the addition of Tamoxifen lead to a roughly three-fold upregulation of promoter activity. Variations in the ratio of the two heterodimerization partners led to an increased inducibility, up to total of 5.3-fold.

Please replace the paragraphs beginning on page 110, line 26, through page 112, line 12, with the following:

DS2
For all transfections, HeLa cells were plated in 24-well dishes and used at a confluency of 40-60%. Typically, 175 ng reporter plasmid (pGL3-promoter constructs or, as negative control, pGL3basic) and 25 ng effector plasmid (zinc finger constructs in pcDNA3 or, as negative control, empty pcDNA3.1) were transfected using the Lipofectamine reagent (Gibco BRL). Cell extracts were prepared approximately 48 hours after transfection. Luciferase activity was measured with the Promega luciferase assay reagent in a MicroLumat LB96P luminometer (EG&G Berthold).

Bombyx mori EcR

A plasmid (LNCVBE) containing the coding region for *Bombyx mori* EcR was obtained from F. Gage. *Bombyx mori* EcR is PCR amplified from this plasmid using the primers listed below and AmpliTaq DNA Polymerase (Hoffmann-LaRoche). Forward and backward primers were chosen to allow construction of the constructs corresponding to FIG. 14 but replacing *Drosophila* EcR by *Bombyx mori* EcR.

(FseI)-BE: (SEQ ID NO: 63)

GAGGAGGAGGGCCGGCCGGAGGCCTGAATGTGTCATACAGGAGCCC

(SfiI)-BE: (SEQ ID NO: 64)

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GAGGAGGAGGGCCCAGGCGGCCAGGCCTGAATGTGTCATACAGGAGCCC

BE-(A_{sc}I): (SEQ ID NO: 65)

GAGGAGGAGGGCGCGCCCCTCCGCCACGTCCCAGATCTCCTCGAG

C7-R-VP16 // C7-E-VP16

This heterodimer was examined on two reporters, one containing 10 C7 sites and one containing 6 2C7 sites, and in two cell lines, HeLa and NIH. In all cases the C7-R-VP16 construct alone showed a high activation of transcription (840-fold) that did not depend on the presence of Ponasterone A. However the C7-E-VP16 construct showed a very little activation of transcription on its own. C7-R-VP16 // C7-E-VP16 together showed the same behavior as C7-R-VP16 alone.

C7-R // E-VP16

In this heterodimer, the activation domain on RXR is dropped to eliminate the basal activation observed above. EcR has no DNA-binding domain to render activation dependent on the presence of DNA-bound RXR. This heterodimer was tested with the 3-finger protein C7 on the 10C7 reporter and with the 6-finger protein E2C on the E2P reporter that contains a single E2P binding site. In both cases no significant activation could be observed.

C7-R // C7-E-VP16

To combine the low basal activity of C7-R // E-VP16 with the high activation seen with C7-R-VP16 // C7-E-VP16, the activation domain on RXR was dropped but the zinc finger protein on EcR was retained. In this set-up, on a 6x2C7 reporter, a 5-fold activation with very low basal activity was observed. Similar constructs using the more powerful VP64 activation domain have also been made.

E2C- ER // ER-VP64

This heterodimeric construct showed 5.3 fold tamoxifen-dependent activation at ratios of 6.7/60 and 2.2/60 of the erbB-2 promoter.

Please replace the paragraph on page 119, lines 5-20, with the following:

D⁵³
A series of different reporter constructs assembled in order to determine the optimal target DNA spacing and orientation of the C2H2 binding sites for transgene induction, C7LBDBS was transfected into HeLa cells and assayed for basal and tamoxifen induced activity on a series of reporter constructs diagrammed above. Reporter constructs were constructed by cloning double stranded oligonucleotides containing the various binding sites into the multiple cloning site of the pGL3Luc reporter. "Response elements" composed of direct, inverted (palindromic), and everted repeats of two C7 binding sites were compared; each response element was separated by two (2) bp except in the control 6 X 2C7, where spacing was 5 bp. Several arrays of directly repeated single C7 sites were tested with various spacing. The data show that direct repeats and everted repeats are preferred over palindromic binding sites. Further, 6 C7 sites, each separated by 2 bp is comparable to the control element of 6 x 2C7, even though it contains only half the number of individual C7 binding sites.

Please replace the paragraph on page 120, lines 8-23, with the following:

D⁵⁴
Protein binding to DNA was analyzed by gel shift assay. The electrophoretic studies used 2C7 recombinant molecular constructs using native PAGE and SDS PAGE analysis of binding to a DNA probe containing six 2C7 binding sites. In this experiment, the 2C7VP16 protein was used as a control and the P32-labeled DNA probe was the 6x2C7 fragment excised from the 6X2C7pGL3Luc. Sufficient 2C7VP protein was added to yield three distinct gel shifted products. When a similar level of protein for the 2C7LBD A, B, and C were applied, only a single weak band was observed. By comparison to the one and two copies bound bands for the 2C7VP16 control, the 2C7LBD band position suggests it is binding as a monomer. Furthermore, the weak level of binding compared to the 2C7VP16 control suggests the DNA binding affinity of the 2C7 domain is significantly reduced in the context of the LBD fusion protein. Results of *in vitro* expressed proteins by SDS-PAGE, indicated equal amounts of

D54 proteins expressed and the expected relative increase in size for the LBD A, B, and C forms.

Please replace the paragraph beginning on page 122, line 29, through page 123, line 10, with the following:

Left end shuttle plasmid construction for ZFP-LBD Fusion Protein Regulators

D55 Shuttle plasmids containing the left viral ITR, CMV immediate early promoter and ZFP-LBD regulator were prepared in the plasmid pAvCVI_x (Figure 26). Note that this vector contains a loxP recombination site just downstream of the poly adenylation sequence. DNA encoding the intact reading frame for the chimeric regulators C7LBD As(G521R), C7LBD Bs(G521R), and C7LBD Bs(G400V) were excised from the appropriate pCDNA constructions, (see figures 4 and 5 for LBD As and LBD Bs constructs respectively) by digestion with restriction enzymes EcoRI and Not I. The ZFP-LBD DNA fragments were modified with Klenow to fill in the restriction site overhangs and blunt end ligated into the EcoRV at bp 1393 site of pAvCvI_x to generate pAvCv-C7LBD As(G521R), pAvCv-C7LBD Bs(G521R), and pAvCv-C7LBD Bs(G400V).

Please replace the paragraph on page 125, lines 4-23, with the following:

In Vitro Regulation with Adenovirus Vectors

D56 The ability to regulate expression of a transgene delivered by an adenovirus vector was demonstrated by the following experiment. HeLa cells were infected with a mixture of two adenovirus vectors, one containing a fusion protein regulator either (Av3-C7LBD-A(G521R) or Av3-C7LBD-B(G52R), the other containing the 6x2C7SV40-luc cassette. To determine the optimal ratio of target vector to effector vector, two different doses of the transgene or target vector (50 or 250 viral particles per cell) at three different ratios of effector vector (50, 250, 750 particles per cell for each target dose) were tested. Twenty four hours after vector transduction, the cells were treated where appropriate with 100 nM 4-OH-tamoxifen. Following an additional 24 hrs incubation, the cells were lysed and assayed for luciferase activity. For the

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D⁵⁶
Av3CV-C7LBD A(G521R) vector, the data indicate relatively low levels of luc expression in the absence of 4-OHT, a strong 4-OHT dependent induction and a dose dependent increase in luc activity as more fusion protein regulator vector is used. At the highest doses (750 particles per cell) of chimeric regulator vector tested, tamoxifen-specific induction of 460 to 560 fold over basal was achieved at target vector doses of 250 and 50 particles per cell, respectively.

Please replace the paragraph on page 126, lines 15-23, with the following:

D⁵⁷
Details of the animal study are as follows. On study day 1, C57Bl/6 male mice were given a total adenovirus vector dose of 2×10^{11} particles via tail vein injection. On day two blood samples were collected, then animals were injected i.p. with 200 μ l of sunflower seed oil containing 5% DMSO and either no, 50 μ g, or 500 μ g of tamoxifen (Sigma # T56448). Blood samples were collected daily for three days following drug administration, and on study days 8 and 10. At the completion of the study, murine endostatin levels were determined by ELISA (Accucyte Kit, Cytimmune Sciences, Maryland).

Please replace the paragraph beginning on page 127, line 4, through page 128, line 5, with the following:

In addition, groups 5 and 6 were similar to groups 3 and 4, but animals received 0.5×10^{11} of the Av3TATA-mEndo vector and 1.5×10^{11} of the C7LBD regulator vector, for a 1:3 ratio of target to effector. Groups 3 – 6 each contained no drug, 50 μ g, and 500 μ g tamoxifen treatment sub-groups.

D⁵⁷
The results showed a dramatic induction of murine endostatin following the day 2 administration of 50 μ g of tamoxifen. The highest level of induction was observed on day 3, the day immediately following drug administration. Compared to the basal level observed on day 3 in the no tamoxifen groups, the C7LBDA(G521R) and C7LBDB(G400V) regulators gave comparable fold induction, approximately 17 fold, and comparable absolute levels of expression, around 1500 ng/ml. In this study, the endogenous murine endostatin levels in an untreated mouse cohort was 20 ± 7 ng/ml. The drug-induced endostatin

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① expression rapidly declines by day 5, three days after drug administration, which is presumably due to the clearance of the tamoxifen and biological half life of the endostatin protein. In contrast, expression in the Av3RSV-mEndo treatment group persists at 200 ng/ml through day 15. In the 1:3 target to effector ratio groups, tamoxifen-induced expression reached 600 – 900 ng/ml, approximately 1/2 the level in the 1:1 ratio cohorts. This result indicates that *in vivo*, the transgene-containing vector, not the fusion protein-encoding vector, is limiting for absolute protein expression. Furthermore, endostatin expression in the animals treated with 500 μ g tamoxifen was comparable to the animals treated with only 50 μ g, indicating that the lower dose of tamoxifen is sufficient to fully activate the As(G521R) and Bs(G400V) regulators. Finally, the comparable low basal level of endostatin observed in the As(G521R) and Bs(G400V) groups suggests that the endogenous level of estrogen in the C57Bl/6 mice is not sufficient to induce the estrogen-responsive Bs(G400V) regulator. An elevation in basal endostatin levels observed at days 3 – 5 appeared to be a non-specific effect resulting from adenovirus vector administration, since the Av3Null vector has an effect similar to the Av3TATA-mEndo containing groups.

Please replace the paragraph beginning on page 128, line 18, through page 129, line 2, with the following:

EXAMPLE 20

Construction and evaluation of the Cys₂-His₂ Zinc finger DBD-ERLBD regulators in Lentiviral Vectors

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① In order to demonstrate controlled gene expression in an integrated vector system, the regulatory system described in Example 19 with the adenoviral vectors were used to develop a series of lentiviral vectors. These vectors contained either the ZFP-LBD fusion protein linked to the immediate early CMV promoter or a regulatable transgene (either eGFP or luciferase) linked to the 6 X 2C7 array of C7 binding sites and either the minimal promoter from SV40 or C-fos TATA. The fusion protein-encoding vector and the regulatable transgene

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D vector can then be used to generate lentiviral vector supernatant. The supernatant can be used to stably transduce human cells either singly or in parallel. Stable cell lines containing the integrated vectors can then be induced with the appropriate activating drug (*e.g.*, 4-OH-tamoxifen) and gene expression is measured as fold induction in the presence and absence of drug.

Please replace the paragraph beginning on page 130, line 19, through page 131, line 2, with the following:

Evaluation of the ZFP-LBD fusion proteins and regulatable lentiviral vectors

Transduction of HeLa cells by inducible lentiviral vectors

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D Subconfluent HeLa cells were transduced with either HIV6X2C7SvLuc or HIV6X2C7TATALuc vector supernatant for 24 hours followed by transduction with HIVAS521R lentiviral vector supernatant. Cells were allowed to recover from infection for 24 hours in fresh culture medium after which 4-OH-tamoxifen (100 or 1000 nM) was added to the culture for an additional 24 hours. Cells were lysed in a standard luciferase lysis buffer, subjected to freeze thaw and analyzed for luciferase activity using a luciferase assay kit (Promega). The results showed that cells infected with either HIV6X2C7SvLuc or HIV6X2C7TATALuc followed by transduction with HIVCMVAS521R resulted in a 13.1 and 11.7 fold stimulation in luciferase activity respectively, when given 4-OH-tamoxifen.

IN THE ABSTRACT:

Please amend the abstract as follows (a marked-up copy of the amended abstract is attached to this Amendment):

Please replace the paragraph on page 139, lines 1-9, with the following:

ABSTRACT

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D Fusion proteins for use as ligand-dependent transcriptional regulators are provided. The fusion proteins include a nucleotide binding domain operatively linked to a ligand-binding domain. They also can include a transcription